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Impact of D181V and A69T on the function of ferroportin as an iron export pump and hepcidin receptor☆

Roman Praschberger [a,1], Melanie Schranz [a,1], William J.H. Griffiths [c,g], Nadja Baumgartner [a], Martin Hermann [b], David J. Lomas [d,g], Antonello Pietrangelo [f], Timothy M. Cox [e,g], Wolfgang Vogel [a], Heinz Zoller [a,*]

[a] Department of Medicine II, Gastroenterology and Hepatology, Anichstrasse 35, A-6020 Innsbruck, Austria
[b] Department of Anaesthesiology, Medical University of Innsbruck, Innrain 66, A-6020 Innsbruck, Austria
[c] Department of Hepatology, University of Cambridge, UK
[d] Department of Radiology, University of Cambridge, UK
[e] Department of Medicine, University of Cambridge, UK
[f] Department of Medicine, University Hospital of Modena, Policlinico di Modena, Via del Pozzo 71, 41100 Modena, Italy
[g] Cambridge University Hospitals NHS Foundation Trust, Hills Rd, Cambridge CB2 0QQ, UK

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ABSTRACT

Mutations in the only known mammalian iron exporter ferroportin cause a rare iron overload disorder termed ferroportin disease. Two distinct clinical phenotypes are caused by different disease mechanisms: mutations in ferroportin either cause loss of iron export function or gain of function due to resistance to hepcidin, the peptide hormone that normally downregulates ferroportin. The aim of the present study was to examine the disease mechanisms of the thus far unclassified A69T and D181V ferroportin mutations. We overexpressed wild-type and mutant ferroportin fused to green fluorescent protein in human embryonic kidney cells and used a ^{59}Fe-assay, intracellular ferritin concentrations, confocal microscopy and flow cytometry to study iron export function, subcellular localization and the responsiveness to hepcidin. While the A69T ferroportin mutation seems not to affect the iron export function it causes dose-dependent hepcidin resistance. We further found that D181V mutated ferroportin is iron export defective and hepcidin resistant, similar to the loss of function mutations A77D and C367X. This indicates that intact iron export might be necessary for hepcidin-induced downregulation of ferroportin. This hypothesis was investigated by studying the hepcidin response under modulation of iron availability. Incubation of wild-type ferroportin overexpressing cells with holo-transferrin increases the hepcidin effect whereas chelating extracellular ferrous iron causes hepcidin resistance. In this study we present data that postulates to classify the D181V ferroportin mutation as loss of function and the A69T mutation as dose-dependent hepcidin resistant and outline a possible causal link between iron export function and the hepcidin effect.

1. Introduction

Ferroportin disease is an inborn error of iron metabolism associated with heterozygous gene defects in ferroportin [1]. Diagnosis is based on the identification of ferroportin mutations in patients with elevated serum ferritin concentrations and increased iron storage in the liver and or spleen and bone marrow. In previous studies, the age of presentation is variable but is rarely made before the 3rd decade of life [2]. Since its first description as an autosomal dominant iron storage disease in a large Italian pedigree in 1999 [3], that was later shown to be associated with heterozygosity for the A77D mutation in the ferroportin gene [4], numerous disease-associated ferroportin mutations have been identified (reviewed in [2]; for more recent reports of ferroportin mutations see [5–10]). Ferroportin disease has highly variable clinical manifestations, where a classical phenotype can be distinguished from a non-classical phenotype on the basis of transferrin saturation and organ distribution of iron overload. In classical ferroportin disease, hyperferritinemia is associated with low-to-normal transferrin-saturation, and intolerance to iron depletion by venesection; iron overload characteristically affects the liver, spleen and bone marrow [11]. In contrast, the non-classical phenotype is characterized by elevated serum ferritin concentrations and transferrin saturation. In non-classical ferroportin disease, excess iron is predominantly stored in the liver, whereas the spleen and bone marrow are unaffected [2].

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* Corresponding author at: Medical University of Innsbruck, Department of Medicine II — Gastroenterology and Hepatology, Anichstrasse 35, A-6020 Innsbruck, Austria. Tel.: +43 512 504 23397; fax: +43 512 504 23309.
E-mail address: heinz.zoller@i-med.ac.at (H. Zoller).

[1] These authors contributed equally to this work.

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In a search for a detailed molecular understanding of the mechanism that causes iron overload in ferroportin disease, the functional consequences of individual mutations have been investigated in a living cell model of the cognate clinical disorder [12,13]. Accordingly, a genotype–phenotype correlation has been recognized in which the classical phenotype is associated with, for example, the A77D ferroportin variant, which induces loss of the iron export function [12]. Impaired iron export function may result from failure to express functional ferroportin at the plasma membrane or, where subcellular trafficking is unimpaired, a defective 'pump mechanism' for ferrous ions. Reports of intracellular retention of A77D have recently been disputed by the finding that in a modified and arguably more physiological overexpression model, the A77D mutant ferroportin was correctly localized to the plasma membrane [14,15]. The only mutation that did not localize correctly to the plasma membrane is the ferroportin truncation mutation C367X, which was used as a control mutant that was created in vitro and has not been identified in a patient with iron overload [15]. In contrast, the non-classical phenotype can be associated with ferroportin mutations that reduce the affinity of hepcidin for ferroportin thereby impairing this action of hepcidin and thus partially mimicking a hepcidin deficiency state. Well conducted genotype–phenotype correlation studies have shown that Y64N, N114H, N144D and C326Y show a severe phenotype with high transferrin saturation and deposition of iron throughout the liver [13]. Under these circumstances, uncontrolled duodenal iron absorption and unrestricted release of iron from macrophages occur.

Hepcidin is synthesized and secreted into the blood by hepatocytes in response to high hepatic iron, elevated transferrin saturation and pro-inflammatory stimuli such as interleukin-6 [16,17]. Hepcidin controls plasma iron concentration and transferrin saturation by direct interaction with ferroportin in a manner which induces its internalization and degradation [18]. A cysteine residue at position 326 of ferroportin is essential for the interaction with hepcidin and this is a plausible explanation for the non-classical phenotype of patients harboring mutations of this amino acid [19]. Although the non-classical disease phenotype has been reported in patients with the N144H and Y64N ferroportin mutations, when studied in vitro, the molecular interaction between hepcidin and these ferroportin variants appears to be unaffected [19]. However, although these residues are not required for hepcidin binding, the internalization is affected by the mutations Y64N and N144H, where the latter exhibited partial hepcidin resistance [11].

In this study, we investigated the functional consequences of two ferroportin mutations, D181V and A69T. While the former ferroportin mutation is associated with classical ferroportin disease and loss of iron export function, the latter was identified in a patient with a non-classical disease phenotype.

2. Materials and methods

2.1. Plasmid construction

The mutations A69T, A77D, N144H, D181V, C326Y, and H507R were introduced by site-directed-mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) into the pENTR221 vector (Invitrogen, Lofer, Austria) with the insert IOH26826. This is a full length cDNA clone of human ferroportin. The resulting vectors as well as the wild-type ferroportin pENTR221 vector were recombined into the expression vector pcDNA/N-EmGFP-Dest (Invitrogen, using the Gateway LR Clonase system). After recombination, the resulting vector was designated pBABS, which has an open reading frame, where mutant or wild type ferroportin is N-terminally fused to GFP under the control of a CMV promoter. Alternatively, the pENTR221 vector with the ferroportin cDNA (IOH26826) was mutagenized to create a read-through mutation of the stop codon, which was then recombined with the expression vector pcDNA/N-EmGFP-Dest. The resulting vector was designated pRASIL and encodes ferroportin N-terminally fused to GFP and a C-terminal V5-tag. For additional control experiments, the C367X mutant was cloned by inverse PCR: briefly, the pENTR221 vector (Invitrogen, Lofer, Austria) with the ferroportin cDNA insert IOH26826 was digested with HindIII, which is a single cutter. The resulting DNA fragment was used as a template for a PCR reaction with the primers 5′-ACC CAG CTT TCT TGT ACA AAG-3′ and 5′-TTT TCG ACG TAG CCA AGT AA-3′. Both primers were 5′-phosphorylated and the resulting PCR product was purified and circularized by self-religation using a T4 ligase (Invitrogen, Lofer, Austria). This resulting construct was a pENTR221 vector that contained a fragment of the ferroportin cDNA containing codons 1 to 366 of the ferroportin open reading frame; this truncation mutant was designated C367X. To generate expression vectors for N-terminal fusion of truncated ferroportin with GFP and N-terminal fusion with the V5 tag, this pENTR211 vector was recombined with pcDNA/N-EmGFP-Dest as described above.

Cell culture, transfection and preparation of ^{59}Fe labeled transferrin were carried out as previously described [5].

2.2. ^{59}Fe export & intracellular ferritin quantification

Twelve hours after transfecting HEK (human embryonic kidney) 293T cells with plasmids encoding wild type or mutant ferroportin fusion proteins (A77D, N144H, C326Y, A69T, and D181V) the effectene–DNA-complexes were removed and 1 ml fresh medium supplemented with ^{59}Fe-holotransferrin corresponding to 200 kBq ^{59}Fe was added to each well. Four wells were left untransfected and served as controls. After incubating overexpressing cells with ^{59}Fe transferrin for 24 h, cells were washed twice with PBS to remove unbound transferrin and 1 ml fresh medium was added. Six hours later, the culture medium was removed and the pellet was collected to determine iron export and iron retained by gamma counting. Each experiment was performed in duplicate and repeated three times. The relative iron release from wild type or mutant ferroportin overexpressing cells or untransfected cells was calculated by correcting for the amount of ^{59}Fe-uptake after 24 hour incubation and expressed as relative to wild-type iron export for each individual experiment. To complement the results from ^{59}Fe export assays, cellular ferritin was quantified as a surrogate for intracellular iron retention. For ferritin ELISA (Abnova, Walnut, CA, USA) cells were seeded in 6-well plates before transfection with plasmids encoding wild type or mutant ferroportin fusion proteins (A77D, N144H, C326Y, A69T, D181V or C326X) for 24 h and incubated for 48 h in the presence of 2 g/l holo transferrin. Cells were then washed with PBS and lysed by the addition of 100 μl lysis buffer (1% Triton X 100 in PBS with cOmplete Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland)). Total protein content was quantified and results from the ferritin ELISA corrected for total protein content.

2.3. Flow cytometry

Twelve hours after transfection with wild type or A77D, N144H, C326Y, A69T, or D181V mutant GFP-coupled ferroportin, 1 ml fresh medium with or without hepcidin (Bachem, Bubendorf, Switzerland) at a concentration of 500 nM was added to each well. Culture medium was also supplemented with 2 mg/ml holo-transferrin (Scipac, Sittingbourne, Kent, UK) to prevent cell death due to iron depletion in this long experimental setting. After 56 h cells were harvested by incubation with Cell Dissociation Solution (Sigma, Vienna, Austria) at 4 °C for 30 min. Flow cytometry analysis was then performed on a FACSCalibur instrument (Becton Dickinson, Vienna, Austria). Mean fluorescence intensity was quantified using the 488 nm laser and a 530 ± 20 nm filter. Raw data were analyzed using the FACS analysis software WinMDI 2.9 (copyright© Joseph Trotter). Hepcidin effects were expressed as relative decrease in mean-fluorescence intensity. The results from three independently performed experiments are shown.

For dose–response experiments, cells were incubated with transfection complexes for 12 h and allowed to recover for 24 h in fresh medium before incubation in serum-free medium and hepcidin at concentrations of 10 nM, 70 nM and 500 nM. After incubation in the presence of hepcidin for 4.5 h, cells were harvested and analyzed by flow cytometry as described above.

To quantify the effect of intra- and extracellular iron modulation on hepcidin response, HEK293T cells were transfected with wild-type ferroportin for 12 h before transfection complexes were removed and cells were further incubated for 24 h in fresh medium supplemented with 2 mg/ml holo-transferrin. Hepcidin effects were quantified by flow cytometry as described above after incubation of cells in serum free medium supplemented with 70 nM hepcidin for 4.5 h. For iron chelator experiments, wild-type ferroportin overexpressing cells were incubated in medium not supplemented with transferrin during the 24 hour recovery period after transfection. To assess the effect of iron chelation on hepcidin mediated ferroportin degradation, the hydrophilic ferrous iron chelator bathophenantroline disulfate (BPS) (Sigma, Vienna, Austria) or the hydrophilic ferric iron chelator deferroxamine (DFO) (Sigma, Vienna, Austria) at a 1 mM concentration was added during the 4.5 hour incubation period with 70 nM hepcidin before fluorescence intensity was quantified by flow cytometry as indicated above.

2.4. Immuno-flow cytometry

To quantify ferroportin at the plasma membrane, HEK293T cells were transfected with the pRASIL vector, where ferroportin is N-terminally fused to GFP and C-terminally fused to the V5-sequence tag. Wild-type as well as A77D, N144H, C326Y, D181V, A69T and C367X ferroportin mutants were overexpressed as V5 fusion proteins. Thirty six hours after transfection cells were dissociated as described above and incubated with a primary mouse anti-V5 antibody (Invitrogen, Lofer, Austria) or an IgG2a isotype control antibody (Abcam, Cambridge, UK) for 1 h. After washing, cells were incubated with a secondary *goat* anti-mouse PE labeled antibody (Abcam, Cambridge, UK) for 45 min. The increase in red fluorescence was quantified on a FACSCalibur instrument using the 488 nm laser for excitation and a 585 ± 21 nm filter for fluorescence detection (Becton Dickinson, Vienna, Austria). Raw data were analyzed with the CellQuest software (Becton Dickinson).

2.5. Confocal microscopy

One day after transfection as described above, HEK293T were analyzed by confocal microscopy as previously described [20].

3. Results

3.1. D181V causes reduced iron export function while A69T does not impair iron export

To study the functional consequences of ferroportin mutations we overexpressed the hitherto unclassified D181V and A69T ferroportin mutants or ferroportin harboring the control mutations A77D, N144H, C326Y or wild-type ferroportin in HEK293T cells. To evaluate the impact of ferroportin mutations on its iron export function, we quantified exported and retained ^{59}Fe in transfected cells and calculated the relative iron export, which we compared with iron exported by cells overexpressing wild-type ferroportin (Fig. 1A). Overexpression of wild type ferroportin in HEK293T cells results in an approximately 5-fold increase in the rate of iron export when compared with mock transfected cells. Cells overexpressing the A69T mutant ferroportin exported iron at a rate comparable to that of wild-type ferroportin expressing cells. The D181V ferroportin mutant is associated with an apparent loss of iron export activity. Cells overexpressing the control mutations N144H and C326Y show normal iron export function in this assay, which is in contrast to cells expressing the control mutation A77D. These findings were confirmed by quantification of intracellular ferritin by ELISA in cells loaded with iron by incubation in the presence of 2 g/l human holo-transferrin. Fig. 1B shows that intracellular ferritin concentrations in HEK293T cells expressing A77D, D181V or the truncation C367X mutant are comparable to the ferritin concentration in extracts from untransfected HEK293T cells. In contrast, ferritin concentration in cells expressing the A69T mutant is decreased to a concentration comparable to that of wild type ferroportin expressing cells. Taken together these results show that the D181V ferroportin mutation causes reduced iron export function, whereas the pathogenic mechanism of the A69T mutation is not apparent from these experiments.

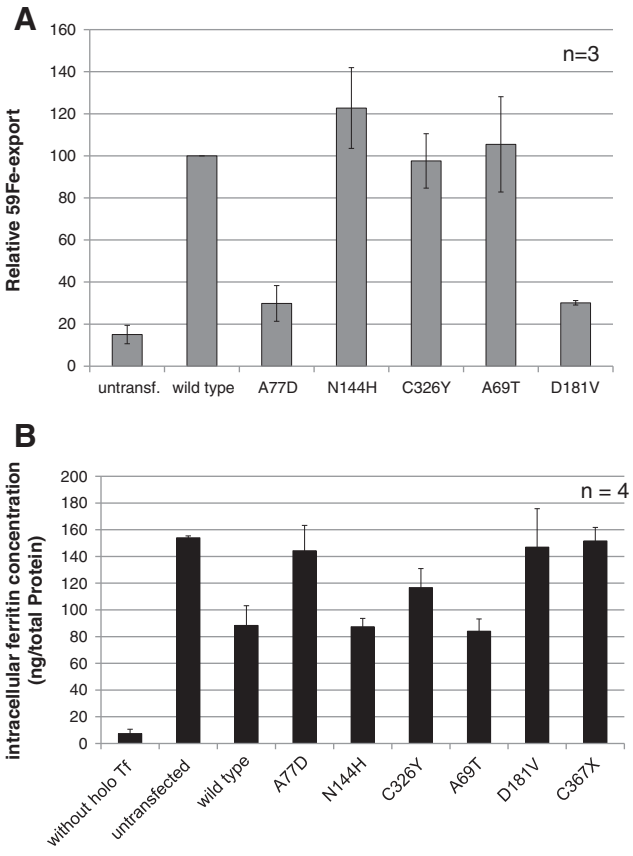
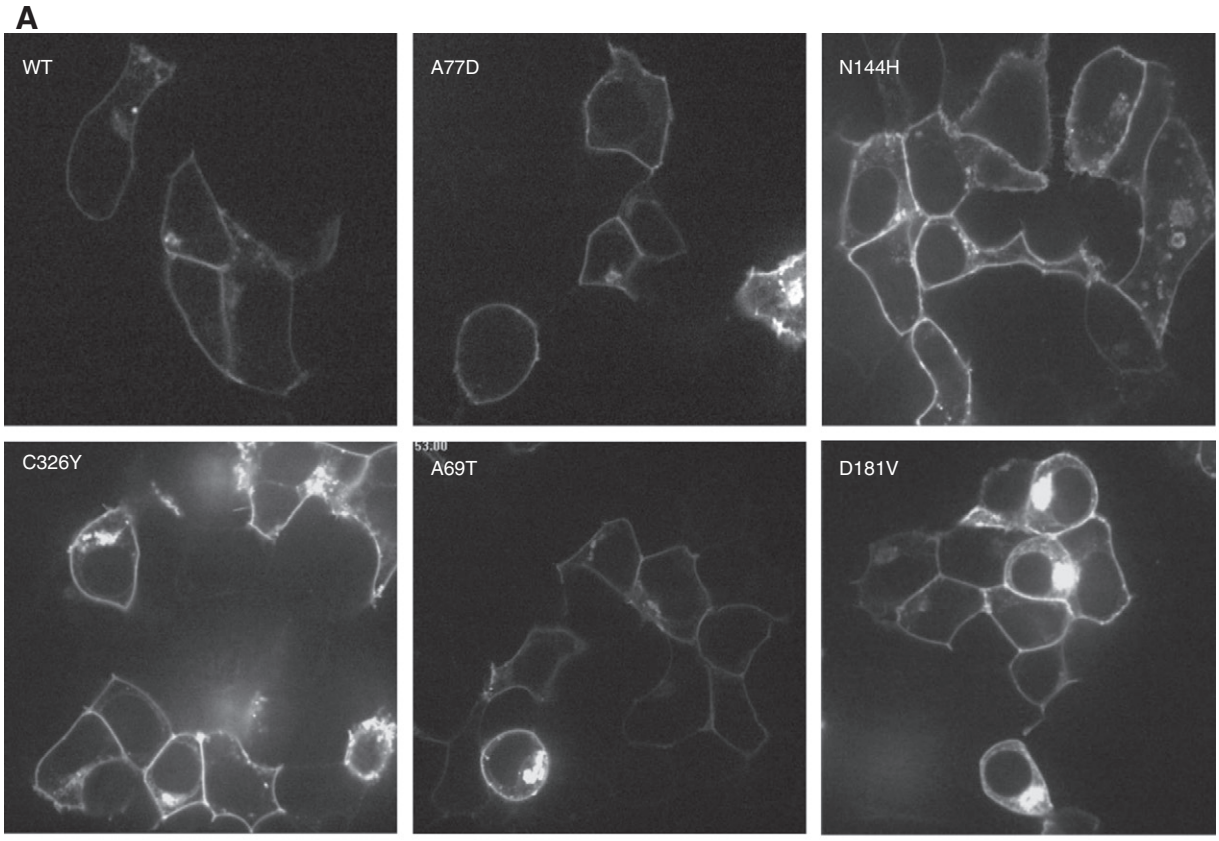


Fig. 1. Relative iron export via wild-type and mutant ferroportin. ^{59}Fe exported within 6 h is expressed as a fraction of total ^{59}Fe uptake and expressed relative to iron exported from wild-type ferroportin overexpressing cells. Wild-type, N144H, C326Y and A69T mutant ferroportin cause a more than 5-fold increase in iron export when compared to untransfected cells. No such increase can be observed in A77D and D181V mutant ferroportin overexpressing cells. Mean and standard deviation of three independently performed experiments are shown. Each experiment was performed in duplicates. *Indicates $p < 0.05$ as tested by a Mann Whitney *U* test when compared to wild type ferroportin expressing cells.

3.2. All studied mutations localize to the plasma membrane

A possible mechanism of how ferroportin mutations might cause reduced iron export is intracellular retention of mutant ferroportin. Only ferroportin correctly localized to the plasma membrane is able to export iron from the cell. To address this question we investigated the subcellular localization of wild-type and mutant ferroportin by confocal microscopy and immuno-flow cytometry (Fig. 2). As shown in Fig. 2A, ferroportin fused with GFP localized to the plasma membrane in cells overexpressing either wild-type ferroportin or the A69T, N144H, C326Y, D181V and the A77D mutants of ferroportin. In addition, a variable amount of intracellularly retained fluorescence is present. To confirm that all investigated ferroportin mutants can traffic to the plasma



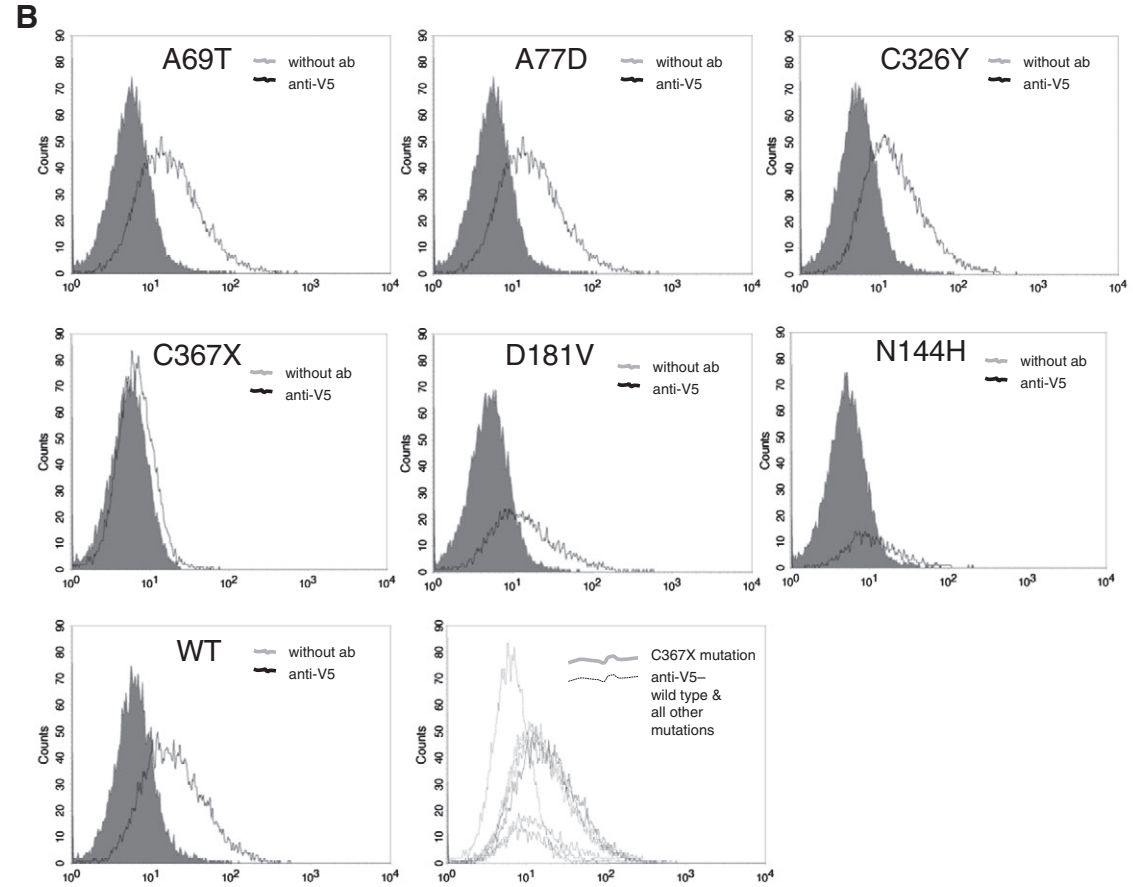


Fig. 2. Plasma membrane localization of wild-type and mutant ferroportin. (A) Confocal microscopy reveals prominent membrane fluorescence of wild-type and A77D, N144H, C326Y, A69T and D181V mutants of GFP-ferroportin. (B) In flow-cytometry studies, the addition of an anti-V5 primary antibody and a secondary PE labeled antibody to non-permeabilized GFP-ferroportin-V5 overexpressing cells causes an increase in red fluorescence (black line) against the background signal (gray histogram). This confirms that wild-type and A77D, N144H, C326Y, A69T and D181V ferroportin mutants localize to the plasma membrane.

membrane, we quantified plasma membrane ferroportin with an additional assay. For this aim, cells were transfected with a plasmid vector encoding ferroportin which is N-terminally fused with GFP and has a C-terminal V5-tag. Fluorescence was quantified by flow cytometry in non-permeabilized wild-type and mutant GFP-ferroportin-V5 overexpressing cells by indirect immunofluorescence using a PE-goat anti mouse antibody and a V5 antibody. As a control the ferroportin truncation mutation (C367X) was expressed and also analyzed by FACS, which shows that this truncated ferroportin variant does not traffic to the plasma membrane. As shown in Fig. 2B all other investigated ferroportin variants are expressed at the cell surface as shown by immune flow cytometry. Histogram data show that the concentrations of ferroportin expressed at the cell surface are comparable between cells expressing different mutations. This finding strongly supports the concept that the apparent reduction in iron export function of D181V and A77D mutant ferroportin is caused by a defective pump mechanism and not by intracellular retention.

3.3. Analysis of hepcidin resistance in different ferroportin variants

To test the response of wild-type and D181V, A69T, A77D, N144H and C326Y ferroportin mutants to hepcidin we used a flow-cytometry assay. We quantified the decrease in mean fluorescence intensity (MFI) of HEK293T cells overexpressing GFP-ferroportin after incubation with 500 nM hepcidin for 56 h. We used a long incubation time and a high hepcidin concentration to study maximal internalization and degradation. To prevent cells from dying due to iron depletion in this long experimental setting we incubated the transfected cells with 2 mg/ml holo-transferrin. As shown in Fig. 3A the addition of hepcidin in a 500 nM concentration for 56 h causes approximately a 55% decrease of MFI in wild-type ferroportin overexpressing cells. A comparable effect is found in A69T mutant ferroportin transfected cells whereas the loss of function ferroportin mutants D181V and A77D and the non-classical control mutation C326Y show a significantly reduced response to hepcidin. The response of N144H mutant ferroportin to hepcidin is slightly reduced when compared to wild type overexpressing cells. To further investigate the response of the disease associated ferroportin mutations A69T and N144H to hepcidin, lower hepcidin concentrations and shorter incubation time were used (4.5 h and three hepcidin concentrations — 500 nM, 70 nM, 10 nM). When incubated with 500 nM hepcidin for 4.5 h no difference in the reduction of MFI in wild-type and A69T and only a slightly reduced effect in N144H ferroportin was apparent (Fig. 3B). However, when we used reduced hepcidin concentrations of 70 nM or 10 nM for 4.5 h the hepcidin-response of A69T and N144H mutant ferroportin overexpressing cells was only half of that observed in wild-type ferroportin transfected cells. Therefore we conclude that the ferroportin mutants A69T and N144H are partially hepcidin resistant.

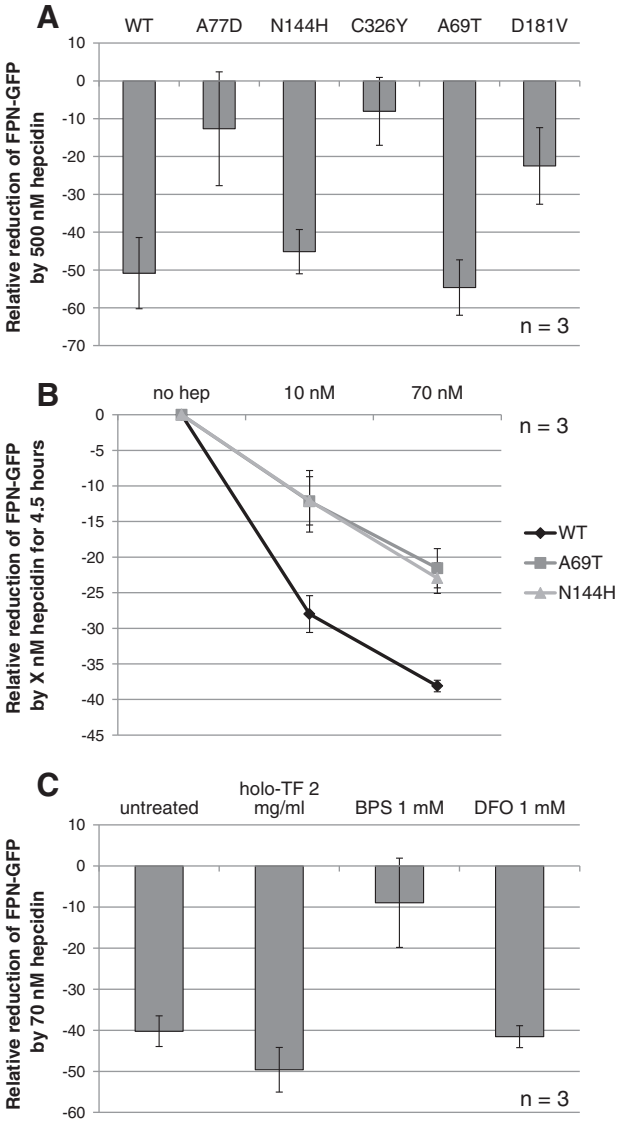


Fig. 3. Differences in dose-dependent downregulation of ferroportin mutants by hepcidin. (A) C326Y, A77D, and D181V ferroportin mutants cause a decreased hepcidin-effect after incubation for 56 h with 500 nM hepcidin. A69T and N144H ferroportin mutants are comparable to wild-type ferroportin. (B) At a hepcidin-concentration of 70 nM or 10 nM for a 4.5 hour incubation period, the hepcidin effect is reduced to approximately 50% when compared with the hepcidin effect in wild-type ferroportin expressing cells. (C) Incubation of cells with 2 mg/ml holotransferrin increases, while chelating extracellular ferrous iron decreases the hepcidin-effect. Mean and standard deviation of three independently performed experiments are shown. *Indicates $p \leq 0.05$ as tested by a Mann Whitney *U* test when compared to wild type ferroportin expressing cells.

3.4. Iron is required for hepcidin dependent ferroportin internalization

Our finding, that the loss of function mutations D181V and A77D also confer a reduced response to hepcidin led us to the hypothesis, that iron transport might be necessary for hepcidin mediated internalization and degradation of ferroportin. To further investigate this question we studied the hepcidin-effect with or without prior incubation with 2 mg/ml holo-transferrin and in the presence of the ferrous iron chelator bathophenantroline disulfate (BPS) or the ferric iron chelator deferoxamine (DFO). As shown in Fig. 3C, significantly more GFP-ferroportin is internalized and degraded in HEK293T cells, which were incubated with holo-transferrin. In contrast, extracellular ferrous iron chelation causes hepcidin-resistance while ferric iron chelation does not. These findings show that iron is required for hepcidin-dependent ferroportin internalization.

3.5. D181V causes the classical, A69T the non-classical disease phenotype

We identified the D181V mutation in an English family with autosomal dominant inherited hemochromatosis (Fig. 4A). The proposita (II.3) presented at aged 52 with a ferritin of 1040 μg/l and a 24% transferrin saturation. Magnetic resonance imaging (MRI) demonstrated both hepatic and splenic iron overload (Fig. 4B). Homozygosity for p.C282Y of the HFE gene was excluded. One of her two sons (III.1) was found to have a ferritin of 932 μg/l and 24% transferrin saturation — the D181V mutation was confirmed. Her other son (III.2) had a ferritin of 228 μg/l with absence of D181V. Two siblings of the proposita had been diagnosed with iron overload and venesected — both harbored the D181V mutation. The brother (II.4) had in fact developed hepatocellular carcinoma and underwent liver transplantation. A liver biopsy from the sister (II.1) was reported as demonstrating slight hepatic but prominent

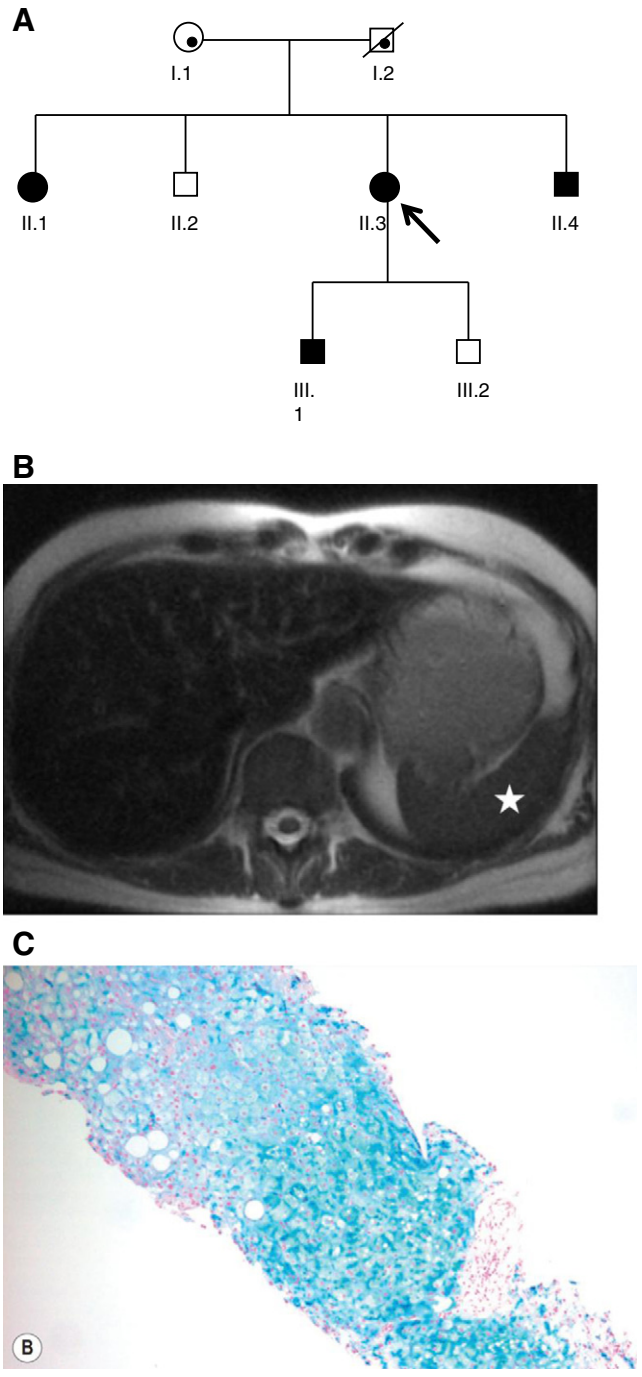


Fig. 4. Pedigree and abdominal MRI of the index case of the family affected by ferroportin disease associated with the D181V ferroportin mutation. (A) Pedigree showing inheritance pattern compatible with autosomal dominant transmission of hyperferritinemia. Shaded boxes represent those diagnosed with evidence of iron overload. Boxes marked with a small dot in the lower right quadrant indicate that this family member was not genetically tested. The index case is indicated by an arrow. (B) The abdominal MRI of the index case shows the reduced signal of the liver, spleen and bone marrow, which indicates iron overload in these organs. (C) Perls' stained liver biopsy showing hepatocellular iron accumulation compatible with non-classical A69T ferroportin disease.
Reprinted from MacSween's Pathology of the Liver, 6th Edition, Disorders of iron overload, Paterson AC, Pietrangelo A., page 271 copyright 2012 with permission from Elsevier [21].

Kupffer cell siderosis. The overall phenotype evident in this family with the D181V mutation is one of classical ferroportin disease. In contrast, a patient from an Italian family harboring the A69T mutation presented with non-classical ferroportin disease: the liver biopsy showed predominantly iron deposition in hepatocytes (Fig. 4C) [21]. The classical disease phenotype of the family with the D181V mutation and the non-classical clinical disease of the A69T family are in line with the classical, loss of function disease mechanism which we observed in vitro for the D181V mutation and the non-classical, hepcidin-resistance mechanism for the A69T mutation.

4. Discussion

The aim of the present study was to functionally categorize the ferroportin variants D181V and A69T. On the basis of our finding that the D181V ferroportin mutant was associated with reduced iron export we propose to classify this protein as a loss-of-function variant — a proposition which accords with the classical expression of disease in the affected members of the pedigree. Cremonesi et al. [22] first reported this mutation in an Italian family who also presented with the classical manifestations of ferroportin disease. We further show that the A69T mutant ferroportin is less responsive to hepcidin at low hormone concentrations. Taken together with the fact that the mutation was initially found in patients with hepatocellular iron overload, A69T was assigned to an association with 'non-classical' ferroportin disease [21]. Differential effects of hepcidin in A69T and wild-type ferroportin overexpressing cells were not found when cells were incubated for 56 h with 500 nM hepcidin. In previous studies high hepcidin-concentrations and long incubation times were used to study the effect of exogenous hepcidin on mutant ferroportin [13,19]. More recent studies showed that lower concentrations of hepcidin and shorter incubation times are sufficient to induce internalization and degradation of ferroportin [18,23,24]. The reported reference concentrations for hepcidin in plasma are approximately 10 nM for healthy men and postmenopausal women and thus far lower than the 500 nM concentration formerly applied in the in vitro cell culture studies [25,26]. We contend that such concentrations are supraphysiological and would be found in plasma only under extreme and exceptional conditions. However, in overexpression models such high concentrations may be necessary to effectively internalize high ferroportin levels in these systems. Based on our findings it will be interesting to study the effect of lower hepcidin concentrations on the internalization and degradation of the hepcidin variants N144D and Q248H, which have previously been reported as 'partially hepcidin resistant' [11].

Of note, our experiments showed that D181V & A77D ferroportin mutants are also resistant to hepcidin, despite the fact that the protein localizes normally to the plasma membrane as shown in the V5-tag immuno-FACS assay. Thus hepcidin resistance cannot be ascribed to faulty trafficking to the plasmalemma. An alternative explanation for the hepcidin resistance that was observed in the loss-of-function mutations A77D and D181V would be that intact iron export function is required for hepcidin binding. In support of this hypothesis, we found that chelation of ferrous iron was associated with a decreased hepcidin effect, whereas chelation of ferric iron had no effect on the hepcidin response. In contrast, when iron stores are augmented, the hepcidin effect of ferroportin increases.

From these findings, we conclude that chelatable ferrous iron, possibly within the ferroportin channel, is required for hepcidin to exert its effect on ferroportin. In accordance with this hypothesis Kono et al. found that co-overexpression of ferroportin and GPI-Cp causes partial hepcidin-resistance [27]. This could be due to the fact that ferrous iron is rapidly removed from ferroportin by oxidation through the co-expressed ferroxidase. Furthermore, hepcidin itself has been suggested to bind iron [28–30]. Based on these findings, one could speculate that hepcidin can only bind to ferroportin while it is actively pumping iron. In such a hypothetical scenario, the interaction of hepcidin and ferroportin could involve direct binding of hepcidin to iron in addition to protein–peptide interactions.

Our finding that C-terminal V5-tagged ferroportin could be immunostained in non-permeabilized cells supports the concept that the C-terminus is located extracellularly. While some groups found that the C-terminus was accessible for antibodies only in

permeabilized cells [14,15,31], others detected the C-terminus also in non-permeabilized cells [12,13,32]. The results obtained with immuno-flow cytometry of non-permeabilized cells show comparable membrane-associated fluorescence in cells expressing wild type and mutant ferroportin.

In conclusion, our results provide the link between the disease-associated ferroportin mutations D181V and A69T and altered function of mutant ferroportin in vitro. The observed functional difference in our model system is in line with the diverse clinical manifestations of ferroportin disease that have been reported. The observation that the hepcidin effect on ferroportin can be modulated by controlling the availability of iron supports the concept that hepcidin targets only those ferroportin molecules that are actively pumping iron. These discoveries could refine our understanding of ferroportin as a hepcidin receptor and permit further elucidation of the genotype–phenotype correlation in ferroportin disease.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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